# Metabolic Arrest in Artemia Embryos Quantified with Microcalorimetric, Respirometric, and Biochemical Measurements

S.C. Hand and E. Gnaiger

## Introduction

Understanding the mechanisms by which metabolic processes of organisms are suppressed and then later reactivated in response to environmental change – e. g., acidotic torpidity, anhydrobiosis, anaerobiosis, hibernation, estivation - poses a formidable challenge to regulatory biologists. In some taxa, the mature adult forms have the ability to enter these states of rest (nematodes, tardigrades, bdelloid rotifers, land snails, freshwater bivalves, earthworms, African lungfish: Bushnell, 1974; Cloudsley-Thompson, 1970; Delaney et al., 1977), while in others, specialized resting forms are common only during earlier stages of life cycles (gemmules of sponges; resting eggs of monogonont rotifers, copepods, and brine shrimp; statoblasts of bryozoans; embryos of annual cyprinodontid fish: Bushnell, 1974; Grice and Gibson, 1975; Crowe and Clegg, 1973; Wourms, 1972). Although the degree of hypometabolism experienced by these forms unquestionably differs, the wide occurrence of dormancy and quiescence suggests to us the possible existence of pleiotropic mechanisms for metabolic and developmental control under such conditions. However, it is becoming clear that multiple solutions are employed by different species, and even by different tissues within a species, to reversibly suppress metabolic rate under unfavourable environmental conditions.

Extreme examples of these metabolic transitions are displayed by the gastrula-stage embryo (cyst) of the brine shrimp *Artemia*. Easily-manipulated and reversible shifts from aerobic development to anaerobic dormancy in post-diapause embryos are characterized by complete cessation of carbohydrate metabolism and intracellular acidification (Busa et al, 1982). Anoxia is common in the hypersaline and often high-temperature lakes into which these embryos are released, and the condition is often prevalent within the thick windrows of cysts (and decaying algal mats) that accumulate along shorelines. Additionally, dehydration of *Artemia* embryos can induce an even more extreme,virtually ametabolic, state of anhydrobiosis. Cysts have the ability to undergo essentially complete desiccation in a fully reversible way, a characteristic shared with other cryptobiotes (Crowe and Clegg, 1973). Due to the wealth of physiological and biochemical data existing for this species and its suitability for laboratory studies, we feel that *Artemia* embryos represent an ideal system for the study of metabolic quiescence.

## Anaerobic Dormancy and Aerobic Acidosis in *Artemia* Embryos

*pH-sensitive control sites of carbohydrate metabolism in Artemia*: The role of intracellular pH in the suppression and activation of cellular metabolism has been documented for both unicellular and multicellular organisms (Busa and Nuccitelli, 1984). Post-diapause embryos (cysts) of the brine shrimp *Artemia* undergo reversible transitions between a metabolically active state of aerobic development and a quiescent condition termed anaerobic dormancy (Busa and

Crowe, 1983). As mentioned above, these transitions are accompanied by large shifts in intracellular pH ($pH_i$) from values of $\geq 7.9$ to as low as 6.3 (Busa et al., 1982). Artificial acidification of the $pH_i$ of aerobically developing cysts to 6.8 by exposure to elevated levels of $CO_2$ (aerobic acidosis) induces a quiescent condition comparable to anaerobic dormancy, as judged by suppression of hatching and oxygen consumption (Busa and Crowe, 1983). This method of increasing the proton concentration would also elevate bicarbonate levels in the embryo. On the other hand, active compensatory alterations in the strong ion difference involving active ion extrusion (Stewart, 1978), as typically displayed by other cellular systems in response to $CO_2$ loading, is improbable due to the striking impermeability of the embryo's outer membrane (permeable only to water and low molecular weight gases: Busa et al, 1982; Morris and Afzelius, 1967). Thus, the above observations coupled with the known influences of pH on enzyme kinetics and protein structural equilibria implicate $pH_i$ as a principal regulator of these modulations in energy metabolism.

Our initial efforts were directed at identifying which specific pathways of energy metabolism were being influenced by pH and at what control points. Since in *Artemia* cysts, pre-emergence development (PED) and metabolism is fueled exclusively by the disaccharide trehalose, carbohydrate metabolism was the likely candidate. Thus, in order to identify the $pH_i$-sensitive control points, we measured the changes in concentration of trehalose, glycogen, glycerol, glycolytic intermediates and adenylate nucleotides that occurred during aerobic development, anaerobic dormancy and aerobic acidosis (Carpenter and Hand, 1986a). During aerobic development, trehalose levels declined while glycogen and glycerol were synthesized. These changes were blocked during both anaerobic dormancy and aerobic acidosis, but were resumed upon return of embryos to aerobic incubation. Evaluation of glycolytic intermediates with crossover point analysis supported the conclusion that the conversion of trehalose to glucose (catalyzed by trehalase) was the first nonequilibrium reaction reversibly inhibited by $pH_i$ during both aerobic acidosis and anaerobic dormancy. Inhibition of the hexokinase and phosphofructokinase reactions was also indicated. If one assumes that all mobilized trehalose that is not converted to glycogen or glycerol is completely oxidized under aerobic conditions, then the 95 % shutdown of trehalose catabolism during aerobic acidosis (Carpenter and Hand 1986a) quantitatively accounts for the large suppression of oxygen consumption seen under these conditions (Busa and Crowe, 1983). Our results provide evidence that $pH_i$ is a primary regulator of carbohydrate catabolism in *Artemia* cysts during aerobic/anaerobic transitions, a conclusion further supported by the observation that a change in energy charge is not necessary to achieve this effect (Carpenter and Hand, 1986a).

*Macromolecular coupling of the proton signal to the metabolic response*: Considering the above information, any mechanism proposed for the pH-induced metabolic transitions in *Artemia* must first explain the proton modulation of the trehalase reaction. We have been unable to obtain evidence for a cAMP-mediated protein phosphorylation that alters trehalase activity. Incubation of embryo extracts with various levels of ATP, cAMP, and the catalytic subunit of beef heart protein kinase had no effect on trehalase activity, nor did incubations of the enzyme with alkaline phosphatase. Alternatively, we have suggested that the shutdown of trehalose mobilization results from a shift in the assembly equilibrium of a hysteretic trehalase (Hand and Carpenter, 1986). Hysteretic enzymes have certain kinetic or molecular characteristics that respond slowly to a rapid change in the concentration of a ligand (for example, protons in the case of *Artemia*

trehalase). Isolated trehalase from *Artemia* embryos exists in two active forms that interconvert when exposed to physiological transitions in pH (Hand and Carpenter, 1986a). This hysteretic interconversion is reversible, requires on the order of minutes for completion, and involves a change in enzyme polymerization. The two states differ two-fold in molecular weight and are distinguishable electrophoretically. Compared to the smaller species, the polymerized form was strongly inhibited by acidic pH, ATP and the substrate trehalose. Thus, the shift in assembly equilibrium toward the aggregated enzyme caused by pH values less than or equal to 7.4 may mediate the arrest of trehalose-fueled metabolism during anaerobic dormancy.

In order to explain the inhibition of hexokinase observed under conditions of anaerobic dormancy and aerobic acidosis we purified the enzyme from the brine shrimp cysts and analyzed the pH-dependent kinetic properties of the enzyme and its reaction mechanism (Rees, Ropson and Hand, 1988). As calculated from our previous work (Carpenter and Hand, 1986a), hexokinase indeed catalyzes a non-equilibrium reaction in *Artemia* embryos. It is thought that a negative crossover point in metabolite concentrations, indicative of enzyme inhibition, is typically not observed at the hexokinase reaction because of product inhibition by glucose-6-phosphate (Rolleston, 1972). However, a clear negative crossover is seen at this step in *Artemia* (Fig. 1). Because the hexokinase inhibition occurs while cellular levels of glucose-6-P are decreasing, other regulatory features must be operative. Similar to yeast and mammalian hexokinases, we have found the *Artemia* homologue to be acutely sensitive to inhibition by aluminum and pH values below 7.0. When pH, substrates, products and aluminum (along with its natural chelators, citrate and phosphate) were maintained at measured cellular levels under anoxia, the vast majority of hexokinase activity was inhibited compared to values under control, aerobic conditions. Thus pH-dependent aluminum inhibition may be an important contributor to the *in vivo* inhibition of this enzyme in *Artemia*.

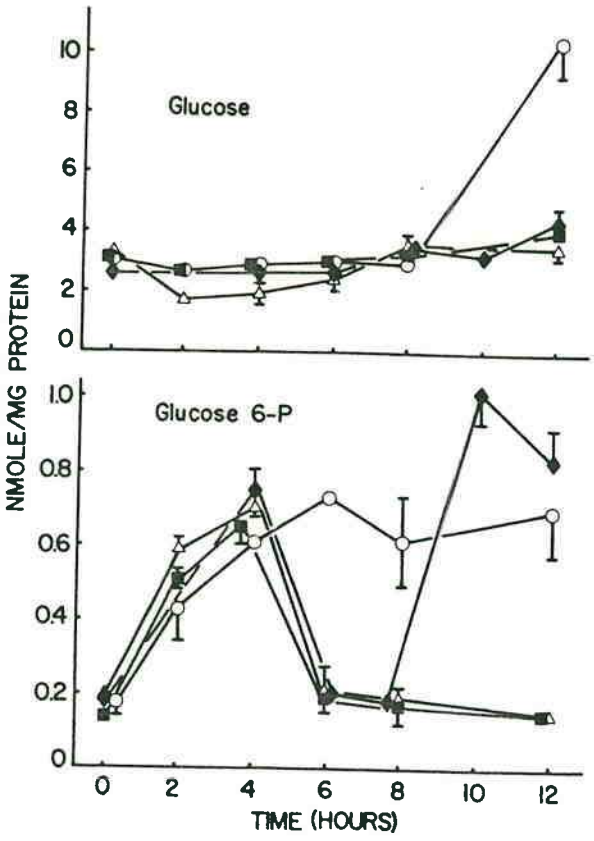


**Fig. 1** Changes in the concentrations of glucose and glucose-6-P during 12- h incubations. Aerobic control (o), anaerobic (Δ ), and aerobic acidosis (■), and reversal of aerobic acidosis (♦). Each point is the mean ± SD. After Carpenter and Hand (1986a).

Investigations centering on *Artemia* phosphofructokinase (PFK) were designed to answer what physical and kinetic properties of the enzyme were responsible for the inhibition of fructose-6-phosphate phosphorylation during intracellular acidification. Consistent with the lack of a Pasteur effect in these embryos (Carpenter and Hand, 1986a), our kinetic measurements with the purified enzyme (Carpenter and Hand, 1986b) showed severe suppression of catalytic activity at acidic pH even when assayed at the

adenylate nucleotide concentrations existing in anaerobic embryos. Based on measurements of 90 °C light scattering, this observed inhibition is not due to pH-induced dissociation of tetrameric *Artemia* PFK into inactive subunits, as is observed with certain other PFK homologues (Hand and Carpenter, 1986b, Hand and Somero, 1983; Hand and Somero, 1984). The contribution of enzyme phosphorylation to the observed pH-dependent inhibition of PFK has not been determined.

*Calorimetric evidence for the control of metabolic switching in Artemia*: Until the application of microcalorimetry, important questions regarding these hypometabolic states have not been experimentally testable due to the lack of adequate methods. First, to what degree is the total energy flux in *Artemia* embryos suppressed during anaerobic dormancy? This point is of particular interest considering the extended periods (weeks) that the organism can survive total anoxia. In this hydrated state, one would expect that some minimal level of metabolism must occur in cysts to provide for cellular maintenance (e. g., DNA repair, protein turnover).

Second, do changes in $pH_i$ that occur under anoxia have modulatory effects on metabolism that are similar to those elicited under aerobic conditions? An affirmative answer to this question is important in demonstrating a regulatory role for $pH_i$ during anoxia. If the shutdown of energy metabolism under anoxia is due directly to intracellular acidification, then artificial alkalinization in the absence of oxygen would be expected to cause an elevation in metabolism.

For the microcalorimetry experiments depicted in Figure 2 (from Hand and Gnaiger, 1988), embryos were perfused with a medium composed of 0.2 mol · $dm^{-3}$ NaCl and 0.05 mol · $dm^{-3}$ tricine-NaOH (pH 8.5 at 23 °C) that was equilibrated with 60 % $N_2$ : 40 % $O_2$. Heat flux continuously increased over the first several hours, as did oxygen flux (data not shown). Towards the end of uninterrupted development as well as after switching to aerobic acidosis, the ratio of heat and oxygen flux was –495 kJ/mol $O_2$, which corresponds well to the theoretical oxycaloric equivalent of –478 kJ/mol $O_2$ for catabolism of carbohydrate under these conditions (Gnaiger, 1983). After 6 h of aerobic perfusion, the gas composition was switched to 100 % nitrogen, which elicited an acute suppression of heat flux (2.4 % of aerobic value after 4 h). This is the lowest percentage thus far reported for euryoxic invertebrates, although it should be noted that the quantitative relationship between calorimetric and biochemical measures of metabolic flux depends on the operative metabolic pathways (Gnaiger, 1983).

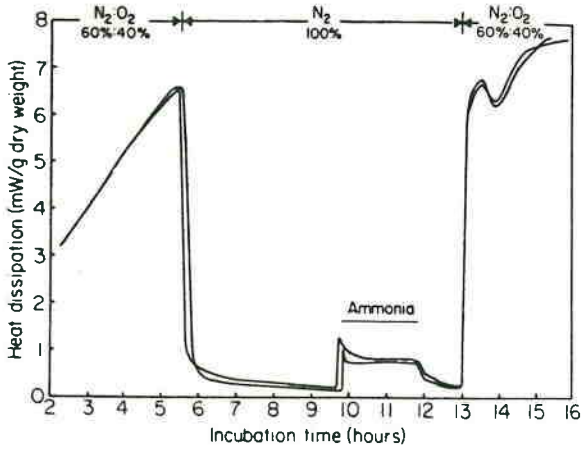


**Fig. 2** Superimposed traces of two indepenent experiments showing heat flux of *Artemia* embryos during aerobic development and anoxia. After Hand and Gnaiger (1988).

The perfusion medium was changed at hour 9.5 to a nitrogen-saturated solution of 0.16 mol · $dm^{-3}$ NaCl, 0.05 mol · $dm^{-3}$ tricine-NaOH that contained 40 mmol · $dm^{-3}$ $NH_4Cl$. This treatment is known to raise the $pH_i$ of embryos approximately 1 pH unit under anoxia (Busa and Crowe, 1983). If the shutdown of energy flux under anoxia is

indeed due to acidification, then this artificial alkalinization should cause an elevated heat flux. A five-fold elevation of heat flux was recorded (Figure 2). The small overshoot at the beginning of the ammonia treatment is quantitatively due to a small pulse of oxygen that was recorded in the inflow at the time of switching to ammonia. At anoxia-steady states, however, oxygen in the system was extremely low (below 0.5 % air saturation; $po_2 < 0.1$ kPa), thus allowing the conclusion that elevation of $pH_i$ under "activated anoxia" results in a deinhibition of energy flux. Our biochemical measurements supported this statement; trehalose utilization is accelerated under anoxia by the ammonia treatment, and at a rate predicted by the elevated heat flux.

The calorimetric data can be used to address an important point regarding the metabolic fuel used for support of this pilot light metabolism of brine shrimp embryos under anoxia. Until this study, the existing hypothesis held that diguanosine tetraphosphate ($Gp_4G$) was the compound used for cellular maintenance during anaerobic dormancy (Stocco et al., 1972). However, based upon the reported rate of $Gp_4G$ catabolism (Stocco et al, 1972) and thermochemical assumptions regarding its degradative pathway (Hand and Gnaiger, 1988), we calculated that $Gp_4G$ metabolism could only explain at most 2 % of the heat flux measured under anoxia across the time frame of our experiment. Consequently we proposed that catabolism of another substrate(s), possibly trehalose, must be contributing to the heat dissipation.

In order to test this hypothesis it was necessary to perform longterm anaerobic dormancy experiments in order to detect any slow decline in the trehalose pool. During a six-day period of anoxia, heat flux continues to decline slowly; the measured rate of trehalose, and to a lesser extent of glycogen catabolism, can account for the vast majority of the energy flux observed under these conditions (Hand, unpublished observations). Gp4G metabolism still explains only a minor portion of the heat dissipation.

The kinetics of recovery from anaerobic dormancy (Fig. 2) displayed a multiphasic pattern, which was not observed during recovery from aerobic acidosis (data not shown). The multiphasic pattern was more pronounced during recovery from anoxic bouts that included an ammonia treatment. Thus, one hypothesis to explain the pattern of metabolic recovery is that the first phase may be a result of aerobic processing of metabolic endproducts (and pre-existing glycolytic intermediates), while the second activation phase may represent the hysteretic activation of trehalase and the resulting mobilization and metabolism of trehalose.

## Anhydrobiosis in *Artemia* Embryos

Analogous to the proton-mediated suppression of metabolism seen under conditions of total anoxia, loss of cell-associated water in *Artemia* embryos induces an even more extreme hypometabolic state of anhydrobiosis. The hydration state of the cysts can be controlled experimentally with some precision (Clegg, 1981) simply by incubating them in NaCl solutions of different concentrations (the cyst is completely impermeable to ions, yet freely permeable to water). The level of metabolism during these transitions appears to be a strict function of water content, providing that molecular oxygen is available. Studies by Clegg and coworkers suggest that there is a critical hydration level (0.6 g $H_2O$/g dry cyst) at which oxygen consumption is initiated, free amino acid mobilization begins, net increase in ATP occurs and trehalose begins to be mobilized (reviewed by Clegg, 1981; 1984). Yet the mechanism by which water uptake initiates these processes in *Artemia* cysts as well as in other anhydrobiotic organisms is not known.

*Regulation points during reductions in cell-associated water*: By applying crossover point analysis of metabolic intermediates, we were able to determine where there were specific regulation points in the carbohydrate catabolism pathways of *Artemia* cysts that were being controlled directly or indirectly by reductions in cellular water (Glasheen and Hand, 1988). When embryos are transferred to oxygenated 5.0 mol · $dm^{-3}$ NaCl solution, a treatment known to reduce cell-associated water into a range referred to as the ametabolic domain (Clegg, 1981), the trehalose utilization and glycogen synthesis that occur during aerobic development of fully hydrated cysts are blocked (Glasheen and Hand, 1988). Upon return to 0.25 mol · $dm^{-3}$ NaCl solution both processes are resumed. Analysis of glycolytic intermediates suggests that the inhibition is localized at the trehalase, hexokinase and phosphofructokinase reactions. ATP levels remain constant during the 6-hour period of dehydration, as does the adenylate energy charge AEC). This pattern is in marked contrast to that observed during anaerobic dormancy, where AEC drops sharply (Fig. 3).

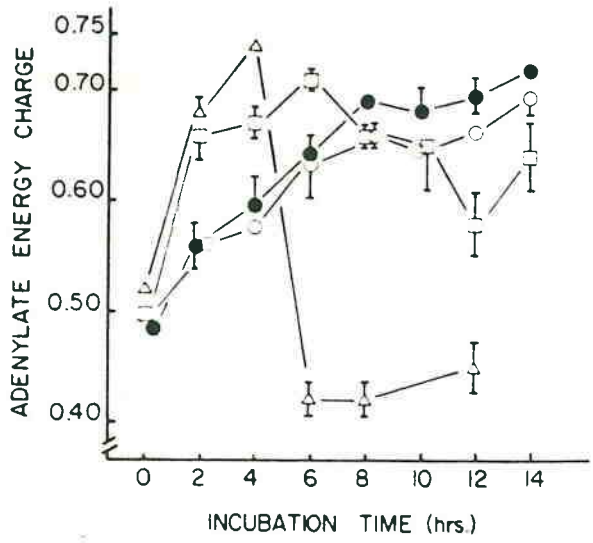


**Fig. 3** Alterations in adenylate energy charge during 14-h incubations of Artemia embryos. Hydrated control (●), dehydrated (○), dehydrated with ammonia (□), and anaerobic dormancy (Δ). Each point represents the mean ± SE. After Glasheen and Hand (1988) and Carpenter and Hand (1986a).

Since acidification of $pH_i$ in fully-hydrated embryos arrests carbohydrate metabolism, we felt it would be informative to dehydrate cysts under conditions where acidification of $pH_i$ (if it were to occur) would be precluded. Thus, an additional dehydration experiment was performed in 5.0 mol · $dm^{-3}$ NaCl solution containing 50 mmol · $dm^{-3}$ ammonium chloride (pH 9.0). Then by comparing to dehydration in the absence of ammonia, one could detect what role, if any, $pH_i$ change plays in the metabolic arrest observed at low water activity.

The metabolic response to dehydration in the presence of ammonia was very similar to the initial dehydration series. Thus, we take these results as strong evidence that the metabolic suppression observed during dehydration does not require cellular acidification, in contrast to the pronounced inhibitory role of low $pH_i$ during entry of hydrated embryos into the quiescent state of anaerobic dormancy. The arrest of carbohydrate metabolism seen during anhydrobiosis indeed appears to be a strict function of embryo water content. The maintenance of high adenylate energy charge in cysts dehydrated for 6 h suggests a water-mediated shutdown of both the energy-consuming biosynthetic machinery and the energy-producing catabolic pathways.

*Microcalorimetric measurements*: Use of flow-through microcalorimetry-respirometry in our studies of anhydrobiosis allow a quantitative appraisal of the extent to which energy flux is reduced during dehydration. Clegg has argued convincingly that because carbohydrate energy stores are not degraded during 28 years of anhydrobiosis in *Artemia* embryos and because respiration is suppressed to immeasurable rates, this physiological state can accurately be termed ametabolic (Clegg, 1981).

Using the open-flow configuration of the microcalorimeter, embryos pre-equilibrated to 0.25 mol · $dm^{-3}$ NaCl can be subjected to increasing concentrations of saline and the heat flux monitored. Kinetics of the transitions into anhydrobiosis under these conditions were on the order of hours rather than minutes as observed for anaerobic dormancy (Glasheen and Hand, unpublished). The heat flux was still slowly declining after 15–20 h of saline hypersaline exposure. However, a clear, stepwise decrease in energy flux was apparent as the NaCl concentration was increased from 1.0 to 2.0 to 3.0 mol · $dm^{-3}$. Further increases to 4.0 and 5.0 mol · $dm^{-3}$ NaCl has little additional inhibitory influence on heat flux.

The molecular mechanism of the arrest may well be explained by perturbation of vicinal water, which results in alterations of metabolic organization and protein polymerization states (Lumry, 1974). It is likely that the enzymatic reactions showing particular sensitivity to cellular hydration state may be those catalyzed by proteins that either reversibly bind to structural elements in the cell or that exhibit regulation through self-polymerization, since such protein conformational changes are known to be quite sensitive to water structure (Clegg, 1981; Clegg, 1984; Lumry, 1974; Yancey et al., 1982). An understanding of the regulatory events involved in desiccation tolerance will require a more precise appreciation of water-protein as well as water-cytoskeletal interactions.

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